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A pyoverdine from *Pseudomonas putida* CFML 90-51 with a Lys ε-amino link in the peptide chain

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Abstract

From *Pseudomonas putida* CFML 90-51 – a hospital isolate – a pyoverdine was obtained which is characterized by the unusual linkage by the ε-rather than the α-amino group of Lys in the peptide chain. The structure elucidation by spectroscopic methods and degradation reactions is reported.

Abbreviations: Common amino acids, 3-letter code; OHAsp – *threo*-β-hydroxy Asp; cOHOrn – *cyclo*-N-hydroxy Orn (3-amino-1-hydroxy-piperidone-2); aThr – *allo*-threonine; Mala – malamide residue; Chr – pyoverdine chromophore (see Figure1); TAP – N/O-trifluoroacetyl (amino acid) isopropyl ester; RP-HPLC – reversed phase high performance liquid chromatography; GC – gas chromatography; ESI – electrospray ionization; FAB – fast atom bombardment; MS – mass spectrometry; CA – collision activation; COSY – correlated spectroscopy; HMBC – heteronuclear multiple bond correlation; HMQC – heteronuclear multiple quantum coherence; NOESY – nuclear Overhauser and exchange spectroscopy; ROESY – rotating frame nuclear Overhauser and exchange spectroscopy; TOCSY – total correlation spectroscopy; DSS – 2,2-dimethyl-2-silapentane-5-sulfonate; TMS – tetramethylsilane; CFML – Collection de la Faculté de Médicine de Lille.

Introduction

Pseudomonas putida is a bacterium commonly found in soil and water. It can use almost any carbon source and it is able to degrade even polycyclic and chlorinated aromatic compounds (Silver *et al.* 1990). It is only potentially human pathogenic, i.e., it may infect persons whose immune system is severely impaired (Graevenitz & Weinstein 1971). Frequently it is associated with the rhizosphere of higher plants belonging to the so-called plant growth promoting rhizobacteria (PGPR) (Kloepper *et al.* 1980). Inoculation of seed material results in large populations on the plant roots, and an increase in the plant growth and crop is observed (e.g., Burr *et al.* 1978). This effect may have several causes, such as the production of antibiotically active substances and the depletion of nutrients for plant deleterious microorganisms, especially of iron (O'Sullivan and O'Gara 1992). *P. putida* is a member of the fluorescent species in the rRNA homology group I of Pseudomonadaceae and produces siderophores with a high complexing constant ('pyoverdines') which for uptake into the cell need special receptors that with few exceptions are available only to the producing strain. Pyoverdines are chromopeptides consisting of the dihydroxyquinoline chromophore, bound amidically to the N-terminus of a peptide chain by its carboxyl group at C-1, and to a small dicarboxylic acid or its amide by the amino group at C-5 (cf. **1**) (Budzikiewicz 1997a, b). The structure elu-

Table 1. ^{1}H-NMR data (δ [ppm]) of **Ib** (pH 4.3; 25 °C; H_2O/D_2O 9:1)[a].

Mala	2′	3′	NH_2							
	2.93	4.68	7.02	7.72						
Chr	1	2a	2b	3a	3b	$4NH^+$	6	7	10	5-NH
	5.63	2.42	2.75	3.40	3.71	N.O.[b]	7.90	7.15	6.98	9.91[c]
Amino acid	NH	α	β	γ	δ	ε	NH_2			
Asp	8.93	4.53	2.62							
			2.81							
Lys[1]	8.05	4.09	1.74	1.22	1.23	2.85	N.O.			
						3.08				
OHAsp	8.70	4.96	4.57							
Ser	8.60	4.51	3.93							
Gly	8.53	4.00								
aThr	8.07	4.28	4.13	1.23						
Lys[2]	8.42	4.32	1.76	1.43	1.67	2.99	N.O.			
			1.88							
cOHOrn	8.36	4.43	1.79	1.83	3.62					
			1.99	1.98	3.66					

[a]Based on COSY and TOCSY correlations.
[b]Not observed.
[c]5 °C.

cidation of the pyoverdine from *Pseudomonas putida* CFML 90-51 showing the rare structural feature of an ε-amino Lys link in the peptide chain will be reported.

Materials and methods

Instruments and chemicals

Mass spectrometry: Finnigan-MAT H-SQ 30 (FAB, matrix thioglycerol/dithiodiethanol), Finnigan-MAT 900 ST (ESI); GC/MS Incos 500 (all Finnigan-MAT, Bremen) with Varian (Sunnyvale CA, USA) GC 3400.

NMR: DPX 300 (^{1}H 300, ^{13}C 75.5 MHz) and DRX 500 (^{1}H 500, ^{13}C 125 MHz) (both Bruker, Karlsruhe). Chemical shifts relative to TMS with the internal standard DSS; δ(TMS) = δ(DSS) for ^{1}H, δ(DSS) = -1.61 ppm for ^{13}C. Suppression of the H_2O signal by the WATERGATE pulse sequence.

UV/Vis: Lambda 7 (Perkin–Elmer, Überlingen), CD: Jasco 715 (Jasco, Tokyo, Japan).

Chromatography: RP-HPLC column Eurospher 100-C_{18} (7 μm) (Knauer, Berlin); low pressure chromatography columns XAD-4 (Serva, Heidelberg), Biogel P-2 (Bio-Rad, Richmond CA, USA), cm-Sephadex C-25 and SP-Sephadex C25 (Pharmacia, Uppsala, S); GC/MS: Chirasil-L-Val (Chrompack, Frankfurt).

Chemicals: Water was desalted and distilled twice in a quartz apparatus; for HPLC it was further purified on XAD-4 resin and filtered through a sterile filter. Organic solvents were distilled over a column. Reagents were of p. a. quality.

Production and isolation of the pyoverdines

Pseudomonas putida CFML 90-51, a hospital isolate from peritoneal fluid, was grown in a succinate minimal medium (Budzikiewicz *et al.* 1997). For the work-up of the culture and isolation of the ferri-pyoverdines by chromatography on XAD-4 and Biogel P-2 see Georgias *et al.* (1999). Separation of ferri-**Ia** and -**Ib** was achieved by chromatography on CM-Sephadex C-25 with 0.02 M pyridinium acetate buffer (pH 5.0). After two minor fractions ferri-**Ia** was eluted. It was decomplexed with 8-hydroxyquinoline (Briskot *et al.* 1986); **Ia** was purified by chromatography on SP-Sephadex C-25 with 0.1 M acetic acid. Control by RP-HPLC gave only one peak. The fourth fraction containing ferri-**Ib** was further separated by chromatography on SP-Sephadex C-25 with 0.02 M pyridinium acetate buffer (pH 5.0). The third (main) fraction was then subjected to preparative RP-HPLC on Eurosphere 100 with a CH_3CN/H_2O gradient (6% CH_3CN for 30 min, than increased to 25%). The second (main) fraction was treated as above.

Table 2. ^{13}C-NMR data (δ [ppm]) of **Ib** (pD 4.3; 25 °C; D_2O)[a].

Mala	1′CO	2′CH_2	3′CHOH	4′$CONH_2$			
	176.2	40.3	69.3	178.2			
Chr	CO	1	2	3	4a	5	6
	170.2	57.9	23.1	36.3	150.2	117.8	140.3
	6a	7	8	9	10	10a	
	115.7	114.9	145.1	153.6	101.5	133.1	
Amino acid	CO	α	β	γ	δ	ε	
Asp	173.7	54.0	39.8	177.5			
Lys[1]	171.7	54.7	31.5	22.3	28.6	40.0	
Ser	173.6	57.3	62.0				
OHAsp	173.2	57.8	73.1	177.1			
Gly	172.5	43.6					
aThr	173.0	60.6	67.8	19.9			
Lys[2]	174.5	54.3	31.1	22.8	27.2	40.3	
cOHOrn	167.5	51.4	27.6	21.1	52.7		

[a]Based on HMBC and HMQC spectra.

For qualitative and quantitative analysis of the amino acids, the determination of their configuration by GC/MS of their TAP derivatives on a chiral column and dansyl derivatization see Briskot *et al*. (1986) and Mohn *et al.* (1990). Partial hydrolysis (see Table 4) was achieved with 6 N HCl at 50 °C for 30 min.

Results

Characterization of **Ib**

The UV/Vis spectra of **Ib** are characteristic for pyoverdines (Budzikiewicz, 1997a and 1997b): 398 nm at pH 7.0, split band at 363 and 376 nm at pH 3.0; ferri-**Ib** 398 nm and broad charge-transfer bands at 470 and 540 nm. The molecular mass of **Ib** was determined by FAB-MS as 1249u. Gas chromatographic analysis of the TAP derivatives after total hydrolysis showed the presence of L-Asp, D-OHAsp, Gly, L-Lys, L-Orn, L-Ser and D-aThr. By total hydrolysis after dansylation α-dansyl Lys as well as ε-dansyl Lys was obtained as could be shown by chromatographic comparison with samples of authentic α- and ε-dansyl Lys. Hence in **1b** for one Lys the α-amino group and for the other Lys the ε-amino group is free. The absolute configuration of C-1 of the chromophore could be determined as *S* from the CD-spectrum (Cotton effect +251 nm, −298 nm, +368 nm) of the 4-hydroxy chromophore obtained by hydrolysis (Michels *et al.* 1991).

Determination of the amino acid sequence

Basis for the sequence determination by NMR is the unambiguous identification of all ^{1}H- and ^{13}C-signals by a combination of homo- and heteronuclear one- and two-dimensional experiments: COSY and TOCSY allows to detect the H-couplings within one amino acid residue (amide bonds interrupt the scalar H,H-coupling). Quaternary C-atoms can be identified with HMBC optimized for ^2J- and ^3J-coupling. Sequence information is obtained by NOESY/ROESY which allows a correlation of an NH-proton with spacially close α- and β-H's of the preceding amino acid (C**H**-C**H**-CO-N**H**) and by HMBC correlating amide-CO with the α-H of the following amino acid (see Figure 1). The ^{1}H- and ^{13}C-data of **1** are compiled in Tables 1 and 2. They correspond to those observed with other pyoverdines (Budzikiewicz 1997a, b). The following ones deserve a comment: The NH-signal of Asp bound directly to the carboxyl group is typically shifted downfield. The shift values of the CH_2-groups of Ser (3.93 ppm) and of the β-CH of aThr (4.13 ppm) show that the OH-groups are not esterified (otherwise an expected downfield shift of about 0.5 ppm would have been observed) (Budzikiewicz 1997b). Also the shift value (4.57 ppm) of the β-CH of OHAsp agrees well with literature data (e.g., Georgias *et al.* 1999). The signals of the α-CH, the ε-CH_2 and the NH of Lys[1] as compared with those of Lys[2] suggest that Lys[1] is not α-peptidically, but rather ε-

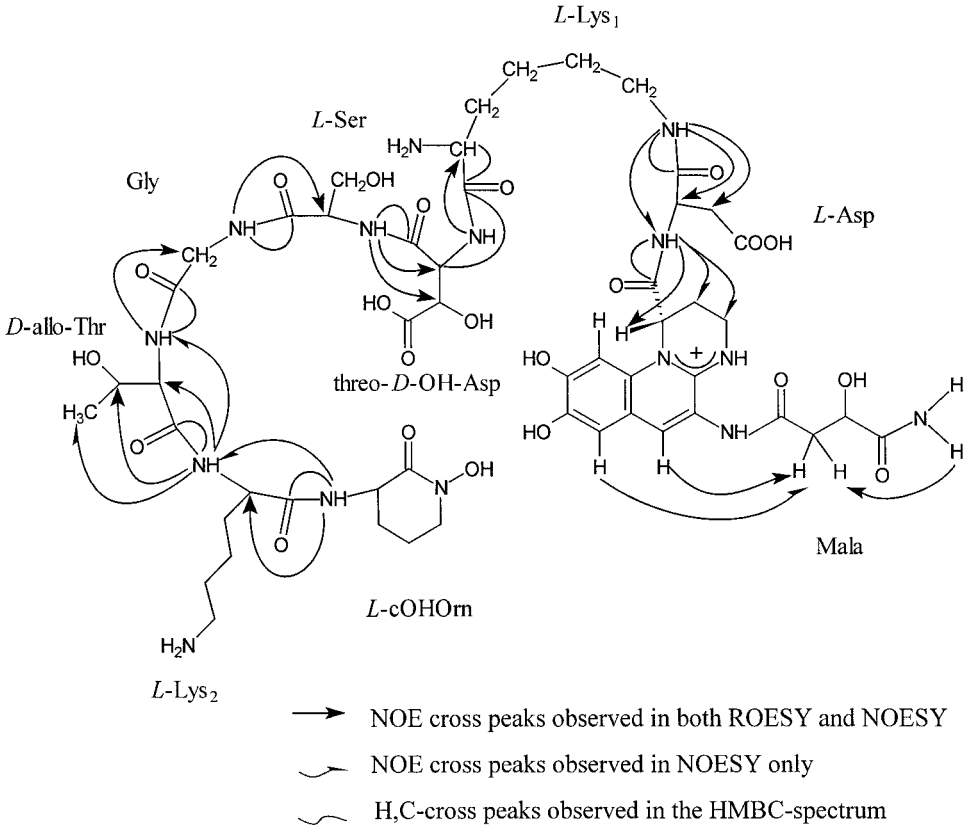

Fig. 1. Sequential information from NMR-data of **1a** (5 °C).

amidically connected to the preceding amino acid (see Discussion below). This is confirmed by NOESY and ROESY cross peaks of the Lys^1-NH-signal (identified by TOCSY and COSY cross peaks with the ε-CH_2 signals of Lys) with the α-, β- and NH-protons of Asp. A signal of the α-NH_2 group of Lys^1 could not be detected due to the faster exchange of amino protons as compared with amide protons. In contrast, the Lys^2-NH shows TOCSY and COSY cross peaks with the α-CH of Lys^2. The different incorporation of the two Lys residues (ε-amidic and α-peptidic) is in agreement with the isolation of α- and ε-Lys (see above). The C-terminal cOHOrn is characterized by the CO-resonance at 167.5 ppm (for peptidically bound Orn the CO resonance is about 174.5 ppm) and by the lower shift values for the ring protons (for reference values see Georgias *et al.* 1999 – cyclic – and Hohlneicher *et al.* 1995 – open). The presence of a malamide side chain and its orientation follows from the shift values and especially from the cross signals depicted in Figure 1. The peptide sequence as derived from ROESY/NOESY and HMBC correlations is given in Figure 1.

Table 3. MS-CA spectrum of **Ib**, B- and Y″-ions.

Amino acid	n	B_n	Y''_n	n
MalaChr	0	373		
Asp	1	488		8
Lys^1	2	616	763	7
OHAsp	3	747		6
Ser	4		504	5
Gly	5	891		4
aThr	6	992		3
Lys^2	7		259	2
cOHOrn				1

The amino acid sequence deduced from NMR data is confirmed by the fragment ions obtained after ESI by CA (Table 3): Most of the so-called B-ions (Roepstorff and Fohlman 1984), viz., X-NH-

Table 4. $[M+H]^+$ ions after partial hydrolysis of **Ib** as determined by FAB-MS.

519	Chr-Asp-Lys
634	Mala-Chr-Asp-Lys
650	Chr-Asp-Lys-OHAsp
765	Mala-Chr-Asp-Lys-OHAsp
852	Mala-Chr-Asp-Lys-OHAsp-Ser
794	Chr-Asp-Lys-OHAsp-Ser-Gly
909	Mala-Chr-Asp-Lys-OHAsp-Ser-Gly
895	Chr-Asp-Lys-OHAsp-Ser-Gly-aThr
1153	Chr-Asp-Lys-OHAsp-Ser-Gly-aThr-Lys-OHOrn
1268	Mala-Chr-Asp-Lys-OHAsp-Ser-Gly-aThr-Lys-OHOrn

$CHR\text{-}CO^+$ are present with high abundance; noticable is the low abundance of the ion B_2 (cleavage after the ε-amidically bound Lys[1;] for a discussion see Budzikiewicz *et al.* 2000). In addition, partial hydrolysis (6 N HCl, 50 °C, 30 min) allowed to identify the degradation products listed in Table 4 by determination of their molecular masses with FAB-MS.

The molecular mass of **Ib** corresponds to the structural details discussed above (amino acids, one cyclic substructure, malamide side chain). NMR data as discussed above in combination with its molecular mass (1234 u) show that **Ia** differs from **Ib** by its succinic acid side chain (NMR: H-2′ 2.73, H-3 2.66; CO 178.2, C-2 32.7, C-3' 32.8, COOH 181.2 ppm).

Discussion

The incorporation of a Lys residue into the peptide chain of a pyoverdin via its ε- rather than via its α-amino group was discussed in detail in a preceding publication (Budzikiewicz *et al*. 1999). This is the first case where Lys appears in both varieties. The NMR ^{1}H shift values fall into the standard ranges given in the above publication. It seems that especially the difference in the NH shifts (ca. 8.4–8.7 for α, ca 8.1–8.2 for ε; at 5 °C slightly higher than at 25 °C) could be an indication for the differing linkage. Nevertheless, chemical proof by degradation after dansylation and availability of both reference substances (α- and ε-dansy-Lsy) is still advisable. It should be noticed also that sofar only *Pseudomonas putida* strains showed the pecularity of ε-Lys linkages in contrast to the large number of pyoverdins obtained from *P. fluorescens* strains. This rare structural feature explains why iron-deficient *Pseudomonas putida* CFML 90-51 is unable to use any foreign pyoverdine for iron transport and why its own pyoverdine when subjected to siderotyping (Meyer *et al.* 1997) shows a specific isoelectrophoretic pattern with three isoforms at pHi 7.35, 6.95 and 5.25, respectively, different from those of the many pyoverdines with known structures (Budzikiewicz 1997b).

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